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13. ABSTRACT (Maximum 200 Words)

The hypothesis driving this project is that effective blocking of tumor angiogenesis by a DNA vaccine encoding the entire VEGF receptor Flk-1 gene induces effective T cell-mediated immunity against proliferating endothelial cells overexpressing Flk-1. This eradicates tumor growth and metastases of RM-2 prostate carcinoma in syngeneic mice. We established that a novel oral DNA vaccine encoding the entire Flk-1 gene successfully suppresses tumor growth and metastases by targeting genetically stable, proliferating endothelial cells in the tumor vasculature rather than mutating tumor cells. This vaccine effectively protected mice from lethal challenges of RM-2 prostate carcinoma cells in a prophylactic model and reduced growth of established metastases in a therapeutic setting. Furthermore, angiogenesis in the tumor vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis, although with a slight delay in wound healing. We also constructed the first anti-angiogenic Flk-1 minigene vaccine and identified the initial H-2 Db-restricted Flk-1 epitope-FLK_{400} (VILTNPISM) specifically recognized by $CD8^+$ T cells. Importantly, these minigene vaccines achieved similar antitumor efficacy as the DNA vaccine encoding the entire Flk-1 gene. Additionally, we elucidated specific CTL-mediated immune mechanisms induced by the Flk-1-based DNA vaccine against RM-2 prostate carcinoma.

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INTRODUCTION

The major aim of this project is to prove the hypothesis that effective blocking of prostate cancer angiogenesis can result in eradication of tumor growth and metastasis of RM-2 and RM-9 prostate cancer cells in syngeneic C57BL/6J mouse tumor model systems. This could be achieved with a novel genomic DNA vaccine encoding either the entire or mini-epitope VEGF receptor *Flk-1* gene that is designed to induce effective T cell-mediated immunity against proliferating endothelial cells in the tumor microenvironment overexpressing *Flk-1*.

It is well known that there are several well-known limitations inherent in strategies designed for direct attacks on tumor cells which achieved only restricted success in cancer immunotherapy. Such strictures include the poor immunogenicity of tumor antigens, the often selective "patient-specificity" of peptide vaccines and gene therapies using dendritic cell approaches. In addition, the genetic instability and heterogeneity of tumor cells frequently produces immune suppressors, down-regulates expression of MHC antigens and generally provides a difficult, moving target for the majority of immunotherapies applied thus far.

For all these reasons, an alternative to a direct attack on tumor cells is required, such as inhibiting tumor growth and metastasis by attacking the tumors' vascular supply. This approach is attractive for several reasons. First, it avoids the development of resistance since the inhibition of tumor-associated angiogenesis is a physiological host mechanism. Second, targeting the tumor vasculature actually potentiates the tumor anti-angiogenic effect since each tumor capillary potentially supplies hundreds of tumor cells. Third, direct contact of the vasculature with the circulation makes for efficient access by therapeutic agents. Fourth, it is well established that angiogenesis plays a key role in the growth and metastasis of solid tumors, where it is a rate-limiting step in the development of tumors that are limited to a growth of 1-2 mm^3 in the absence of a blood supply. In this regard, the vascular endothelial growth factor receptor 2 (FLK-1) which binds all five isomers of murine vascular endothelial growth factor (VEGF) has a restricted expression on endothelial cells (EC). Importantly, FLK-1 is markedly upregulated by hypoxia and once these EC proliferate during angiogenesis in the tumor vasculature. For these reasons, FLK-1 is strongly implicated as a therapeutic target, especially since it is

necessary for tumor angiogenesis and known to play a key role in tumor growth and metastasis. Indeed, several approaches were used by a number of investigators to block FLK-1, including dominant-negative receptor mutants, germ line disruption of VEGF-R genes, anti-VEGF monoclonal antibodies and a series of synthetic RTK inhibitors.

The data obtained during the past three year grant period clearly indicate that we could produce a novel and unique DNA vaccine targeting either the entire FLK-1 gene or selected epitopes within minigene vaccines, all of which can evoke a robust CTL response that effectively suppresses growth and dissemination of prostate carcinoma in a mouse prostate cancer model. The initial focus of our studies was on demonstrating that an effective suppression of RM-2 and RM-9 murine prostate tumor growth can be achieved by this DNA vaccine in syngeneic C57BL/6J mice. Second, we not only demonstrated that a FLK-1 based minigene vaccine could effectively suppress lung metastases of RM-9 prostate carcinoma but also identified one of six $H\text{-}2^b$ peptides as an effective and specific epitope for T cells in this regard. Finally, we demonstrated that immunizations with either, the vaccine encoding the entire FLK-1 gene or a minigene vaccine were truly effective as they both induced an immune response characterized by activated CTL cells and suppression of angiogenesis resulting in suppression of prostate cancer growth and metastasis. Taken together, we developed strategies which allowed us to finish most of our tasks, albeit with some modifications. The following is a summary of results achieved during the grant period with emphasis on the third year of work.

BODY

Task 1: The task outlined in the initial grant proposal was mostly completed. Importantly, proof of concept was established for the highly effective anti-angiogenic/anti-tumor effects of a DNA vaccine encoding the entire murine VEGF receptor 2 (FLK-1). The results obtained are documented in principle in a paper published in NATURE MEDICINE (Niethammer et al., 2002). Second, metastatic prostate cancer models were established and standardized and novel DNA vaccines encoding FLK-1 and CD40LT as well as their modified versions were constructed (Months 5-8) and were critically evaluated for their anti-angiogenic activity (Months 9-15) (Fig 1.). Third, we demonstrated the efficacy of our vaccine carrier consisting of doubly attenuated *Salmonella typhimurium* (DAM^-; $AroA^-$) by showing that they specifically transferred the expression vector encoding FLK-1 to Peyer's patches in the small intestine of mice (Fig. 2). Fourth, work on Task 2 (Months 15-19) has been mostly completed as we finished constructs of minigene-based DNA vaccines encoding multiple FLK-1 nonapeptides with either H-$2K^b$ and/or H-$2D^b$ anchor residues. These constructs are depicted schematically in Fig. 3 and Table 1. The data obtained thus far provide evidence for the suppression of prostate cancer growth and metastases (Fig. 4 and 5). Finally, the results published in our NATURE MEDICINE paper in December, 2002, which established proof of concept for the hypothesis driving this project, were recognized widely, at both the national and international levels and stimulated numerous comments by science writers in leading newspapers, magazines and scientific journals throughout the world (see Appendix). In addition, we extended our research to test effects of a DNA vaccine encoding mouse endoglin combined with secretory IL-15 in a prostate cancer model. The immunization strategy and preliminary data of this project are shown in Table 2.

Task 2: This task, (Months 16-24), outlined in the initial grant proposal and in the revised SOW, was mostly completed. The hypothesis was validated that a FLK-1 based DNA minigene vaccine can effectively suppress growth and hepatic as well as pulmonary metastases of murine RM-9 prostate carcinoma in a syngeneic tumor model in C57BL6/J mice. This was achieved most effectively by the induction of a robust $CD8^+$ T cell response evoked by a polyubiquitinated DNA minigene vaccine with H-$2D^b$ anchors that

also co-expressed CD40LT. The H-2D^b peptide epitopes were fused with the HIV tat peptide to enhance its immunogenicity. Experimental groups of mice immunized with controls such as empty vector (pUB) showed no tumor-protective response and vaccines which only encoded either FLK-1 or CD40LT also proved considerably less effective in evoking a tumor-protective immune response. In addition, minigene vaccines encoding peptides with H-2K^b anchor residues proved to be quite ineffective in suppressing tumor growth as did vaccines encoding H-2D^b peptides 1 and 3. Importantly, the minigene vaccine encoding the H-2D^b peptide 2 was most effective not only in suppressing growth and metastases of RM-9 prostate carcinoma but also in evoking a robust CD8$^+$ CTL response which effectively killed endothelial cells expressing FLK-1. This particular minigene vaccine also induced highly activated CD8$^+$ CTLs as evident from their production of proinflammatory cytokine IFN-γ both at the intracellular and single T cell level.

The construction of the various FLK-1-derived minigenes is shown schematically in Fig. 6. The suppression of RM-9 prostate tumor growth by both a DNA vaccine encoding the entire FLK-1 gene and by various H-2D^b and H-2K^b minigene vaccines is depicted in Fig. 7. The efficacy in suppressing both lung and liver metastases of RM-9 prostate carcinoma is clearly demonstrated for H-2D^b-based DNA minigene vaccines (Fig. 8). The cytotoxicity induced by the various minigene vaccines and the vaccine encoding the entire FLK-1 gene is indicated in Fig. 9 as evident from the specific lysis produced against endothelial cells expressing FLK-1. The superior specific cytotoxic killing activity of CTLs induced by the H-2D^b minigene encoding peptide 2 against endothelial target cells expressing FLK-1 is clearly indicated since whole tumor cells lacking FLK-1 expression were not killed at all (Fig. 10). Finally, Fig. 11 clearly demonstrates that the H-2D^b minigene encoding peptide 2 is also most effective in inducing highly activated T cells as indicated by the production of IFN-γ at both the intracellular level and the single T cell level.

Task 3: As described in our initial grant proposal, we have finished almost all experiments remaining in Task2 that were mentioned in the second fiscal year report. These include the determination of anti-angiogenic/anti-tumor effects of minigene-based DNA vaccines encoding *Flk-1* nonapeptides with either H-2K^b or H-2D^b or both anchor

residues as well as the simultaneously evoked CTL-mediated immune response induced by the DNA vaccine against RM-9 prostate carcinoma outlined in Task 3. In addition, we also extended our research project to include not only the entire FLK-1 gene and minigene vaccines, but also the major domains of the FLK-1 gene. Thus the expression vectors encoding intracellular and cytoplasmic domains, as well as intracellular and extracellular domains of the FLK-1 gene, namely pID/CD and pID/ED respectively were generated and tested in the RM-9 prostate cancer model to identify the most immunogenic antigen domains. The rationale for using the RM-9 instead of the RM-2 prostate cancer model in task 3 is based on the fact that RM-2 is far more aggressive than RM-9, which makes such experiments including the life span far more difficult. In addition, we also found during the course of these experiments that the HI peptide is more effective in the minigene as an immunostimulatory adjuvant and shuttle peptide than CD40LT. However, this is not the case for the vaccine encoding the entire FLK-1 gene or its major immunological domains.

Initially, we assessed the *in vivo* anti-tumor efficacy of a genomic DNA vaccine encoding variable domains of the FLK-1 gene and combined these with CD40LT as a booster (Table 3). The results obtained indicated that both pID/CD and pID/ED can sufficiently suppress RM-9 tumor growth in syngeneic C57BL/6J mice when compared with controls. However, the plasmid encoding pID/ED fragment achieved a better effect in suppressing RM-9 tumor growth than the pID/CD vaccine. Most importantly, the combination of FLK-1 and CD40LT in vaccines resulted in the most efficacious suppression of tumor growth. Second, we modified the design of the minigene expression vectors and evaluated the *in vivo* anti-angiogenic/anti-tumor activities of the DNA vaccines, including minigene vaccines, in an experimental RM-9 prostate carcinoma metastasis model in syngeneic C57BL/6J mice. The emphasis of these experiments was on eradication of tumor growth and metastases as well as the prolongation of life span (Fig. 12, 13). Third, we evaluated the possible immunological mechanisms responsible for the effective immunization with the *Flk-1*/HI minigene vaccine. The focus was on T cell function involved in tumor protective immunity with emphasis on T cell specific cytotoxicity and insight into possible mechanisms (Fig. 14, 15). In this case, the DNA minigene vaccine pHI-Db induced specific killing of the

FLK-1^{+} endothelial cell line MS1, but not of FLK-1^{-} tumor cells, RM-9. Importantly, we developed the first anti-angiogenic minigene vaccine against murine prostate cancer and identified its most effective H-2 Db-restricted FLK-1 epitope, FLK_{400} (VILTNPISM) (Fig. 16) .

KEY RESEARCH ACCOMPLISHMENTS:

1) We have successfully generated all expression vectors encoding the entire FLK-1 gene and some of its minigenes as well as CD40LT that served to construct DNA-based our vaccines. In addition, we also extended our research by designing expression vectors encoding either intracellular domains together with cytoplasmic domains or intracellular domains together with extracellular domains of FLK-1. Furthermore, the experimental metastasis models for RM-2 and RM-9 murine prostate carcinoma were established and standardized in order to critically evaluate the efficacy of the FLK-1/CD40LT, pID/CD and pED/ID as well as minigene-based FLK-1 vaccines in suppressing or eradicating prostate tumors and their pulmonary metastases.

2) We demonstrated that an oral DNA vaccine encoding either the entire FLK-1 gene or defined portions of this gene, can exclusively target genetically stable proliferating endothelial cells in the prostate tumor vasculature due to its upregulated expression of FLK-1. This vaccine when combined with CD40LT, effectively protected mice from lethal prostate cancer cell challenges and reduced prostate tumor growth and metastases in both prophylactic and therapeutic settings.

3) FLK-1 minigenes encoding H-2K^b/D^b epitopes were employed to identify specific CTL epitopes for in-depth mechanism studies, and to tailor such vaccines for optimal T cell activation. We reported the first anti-angiogenic minigene vaccines targeting murine prostate cancer and identified the most effective H-2 Db-restricted FLK-1 epitope, FLK_{400} (VILTNPISM). Importantly, the pHI-Db and pHI-FLK_{400} minigene vaccines achieved similar anti-tumor efficacies as the DNA vaccine encoding the entire FLK-1 gene, and thus offer a more simple and manipulatable alternative strategy.

4) We established that DNA vaccine-induced anti-tumor effects were due to the activation of MHC class I-restricted $CD8^+$ T cells and their increased secretion of the proinflammatory cytokine, IFN-γ and upregulation of co-stimulatory molecules B7.1/B7.2, ICAM-I as well as T cell activation markers CD28, CD25, LFA-1 and CD69. This included also cytokines produced by DCs and T cells, such as IFN-γ and IL-12. Additionally, we demonstrated that effective angiogenesis in the tumor vasculature was markedly suppressed by our DNA-based vaccines without impairment in fertility, neuromuscular performance or hematopoiesis and only a slight delay in wound healing.

REPORTABLE OUTCOMES:

1) Two manuscripts which describe the results summarized here are currently in preparation (For details, please see appendix). The manuscript titles are as follows:

a) A FLK-1 minigene DNA vaccine protects mice from tumors of different origins by inducing immunity and anti-angiogenesis.

b) A DNA vaccine targeting FLK-1 activates CTL and suppresses angiogenesis resulting in eradication of prostate cancer.

2) In addition, funding from this award also stimulated a new research project based on a novel DNA vaccine encoding murine endoglin carried by doubly attenuated *Salmonella typhimurium*.

CONCLUSIONS:

The key finding obtained during this grant period was the proof of concept established that our FLK-1-based DNA vaccines could eradicate growth and metastasis of murine prostate cancer. We proved the hypothesis driving this project to be valid, namely that T cell mediated killing of proliferating endothelial cells lining blood vessels in the tumor vasculature which overexpress FLK-1, leads to the effective suppression of prostate tumor angiogenesis and results in marked suppression of their growth and metastases. A key finding of critical importance was that FLK-1 vaccines proved effective in protecting against growth and metastases of murine prostate cancer by combining the action of immune effector cells with suppression of tumor angiogenesis. Furthermore, we established for the first time that a FLK-1 based DNA minigene or vaccines encoding extra- and intracellular domains of FLK-1 are capable of evoking a $CD8^+$ T cell response which is sufficiently robust to induce the suppression of growth and metastases of murine RM-9 prostate carcinoma. Together with the results from our previous studies on the DNA vaccine encoding the entire FLK-1 gene, these data strongly support the contention that the murine VEGF-receptor 2, which is overexpressed on proliferating endothelial cells in the tumor vasculature, is indeed an effective target for a DNA vaccine against prostate carcinoma. Taken together, our strategy circumvents problems in solely targeting genetically unstable tumor cells. This approach may provide a new approach for the rational design of therapies for the prevention, growth and dissemination of prostate cancer.

APPENDICES:

1) Publications:

a) A FLK-1 minigene DNA vaccine protect mice from tumors of different origin by inducing anti-angiogenesis immunity. He Zhou[1], Yunping Luo[1], Masato Mizutani[1], Noriko Mizutani[1], Ralph A. Reisfeld[1], and Rong Xiang[1,2]

[1] Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

[2] Requests for reprints should be addressed to Rong Xiang, at The Scripps Research Institute, R218, IMM13, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 784-8124; Fax: (858) 784-2708; E-mail: rxiang@scripps.edu.

Running Title: anti-angiogenic minigene protects mice from tumor

The abbreviations used are: Ag, antigen; CEA, carcinoembryonic antigen; E:T, effector: target.

Abstract:

Our laboratory first reported that an oral DNA vaccine against FLK-1 effectively prevents angiogenesis and inhibits tumor growth largely by CD8 T cell mediated immune responses. Minigene approaches were successfully employed to identify specific CTL epitopes for in-depth mechanism studies, and to tailor the vaccine for optimal T cell activation. Here we report the first anti-angiogenic minigene vaccine against murine prostate cancer and identified the first H-2 Db-restricted FLK-1 epitope-FLK_{400} (VILTNPISM). Importantly, the pHI-Db and pHI-FLK_{400} minigene vaccines achieved similar efficacy as the DNA vaccine encoding the full length FLK-1, offering a much simpler and more manipulatable alternative to the whole gene vaccine strategy and adding a new dimension to anti-angiogenic intervention.

b). A DNA vaccine targeting FLK-1 activates CTL and suppresses angiogenesis resulting in eradication of prostate cancer. (Manuscript in preparation)

c). Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertl, U., Karsten, G., Eliceiri, B.P. and Reisfeld, R.A. A DNA vaccine against vascular endothelial growth factor receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat. Med., 8:1369-1375, 2002.

2) Figures

3) Tables

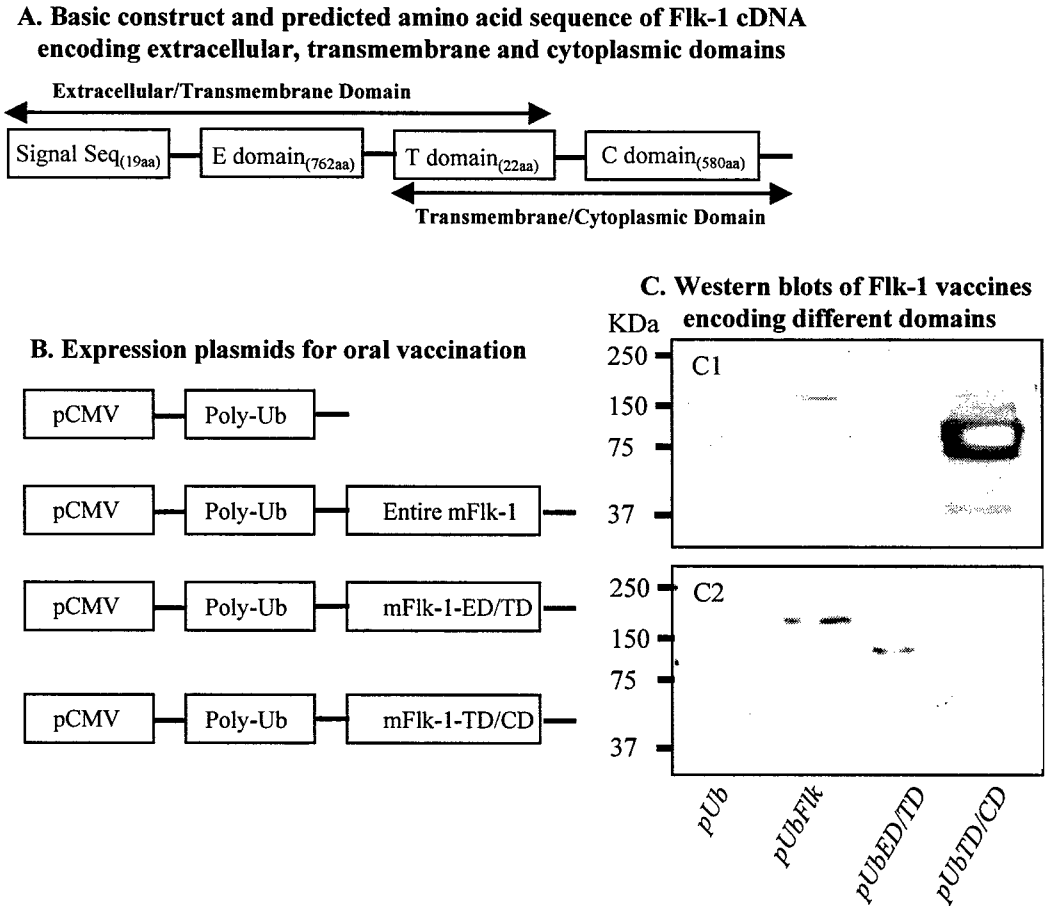


Fig1. Schematic diagram of Flk-1 gene and protein expression. In Fig.1A, the entire Flk-1 gene is shown including each of its bio-functional domain. DNAs encoding the whole Flk-1 gene or different domains were inserted into the pBud vector at the C-terminal of poly-ubiquitin for proper antigen procession and presentation (Fig1. B.). The constructs were verified by nucleotide sequencing and Western blots, shown by Fig.1 C. The results of Western blot were confirmed by using either anti-Flk-1 C-terminal antibody(C1) or anti N-terminal antibody (C2).

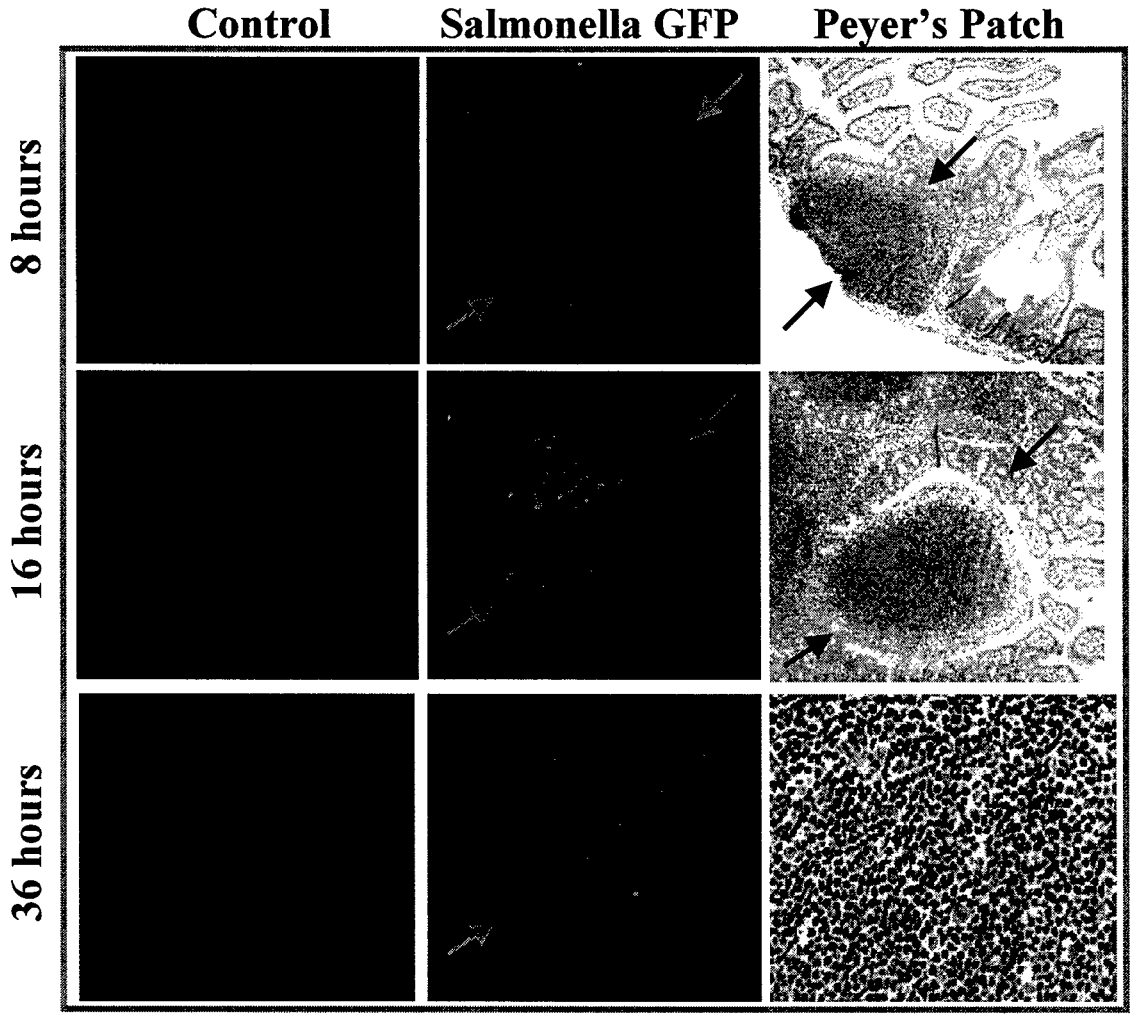
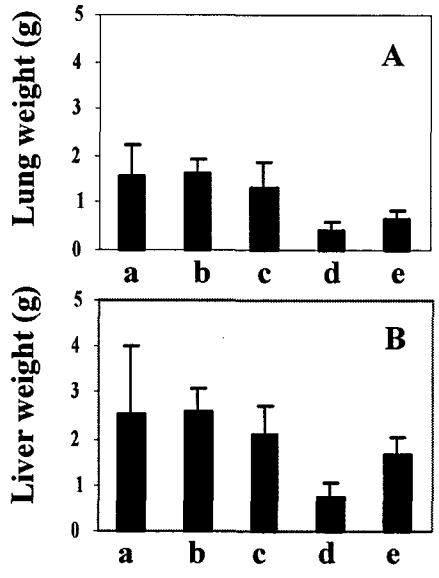


Fig. 2 Evidence for EGFP expression and transfer of expression vectors from double attenuated Salmonella typhimurium to mouse Peyer's Patches.

Fig.3 Schematic representation of Flk-1 minigene-based DNA vaccine expression constructs:

H-2D^b construct:

H-2K^b construct:



a: PBS; **b**. Vector; **c**. HIV; **d**. H-2D^b; **e**. H-2K^b

Fig. 4 Growth suppression of prostate tumors by an oral Flk-1 minigene-based DNA vaccine in both lung and liver syngeneic C57BL/6J mouse model

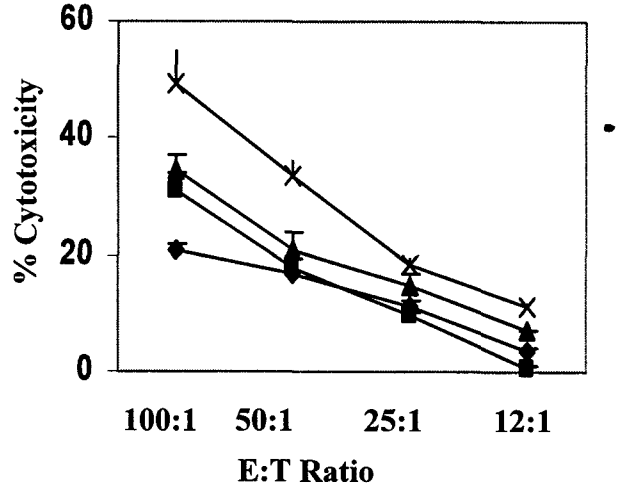


Fig. 5 CTL mediated tumor protective immunity induced by Flk-1 minigene-based DNA vaccines against prostate cancer. (◆) : vector; (■): HIVtag; (×): H-2D^b; (▲): H-2K^b;

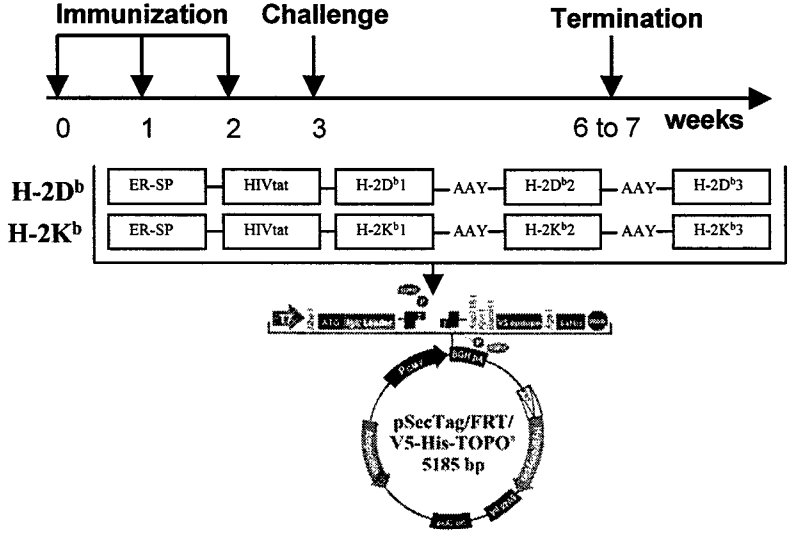


Fig6. Representative diagram for FLK-1 minigene vaccines

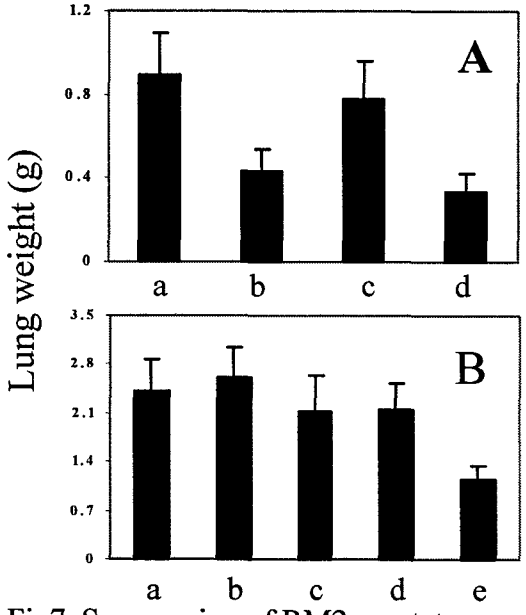


Fig7. Suppression of RM2 prostate cancer growth by entire FLK-1(A) or FLK-1 minigene vaccines(B).

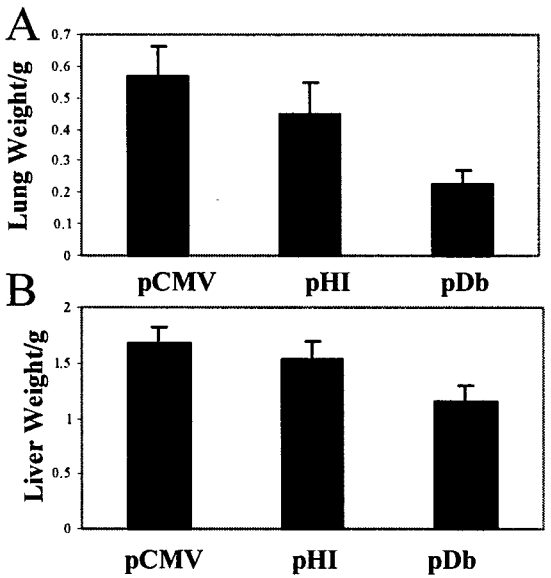


Fig.8 Efficacy of H-2D^b minigene vaccine against prostate cancer RM9 in both lung and liver models

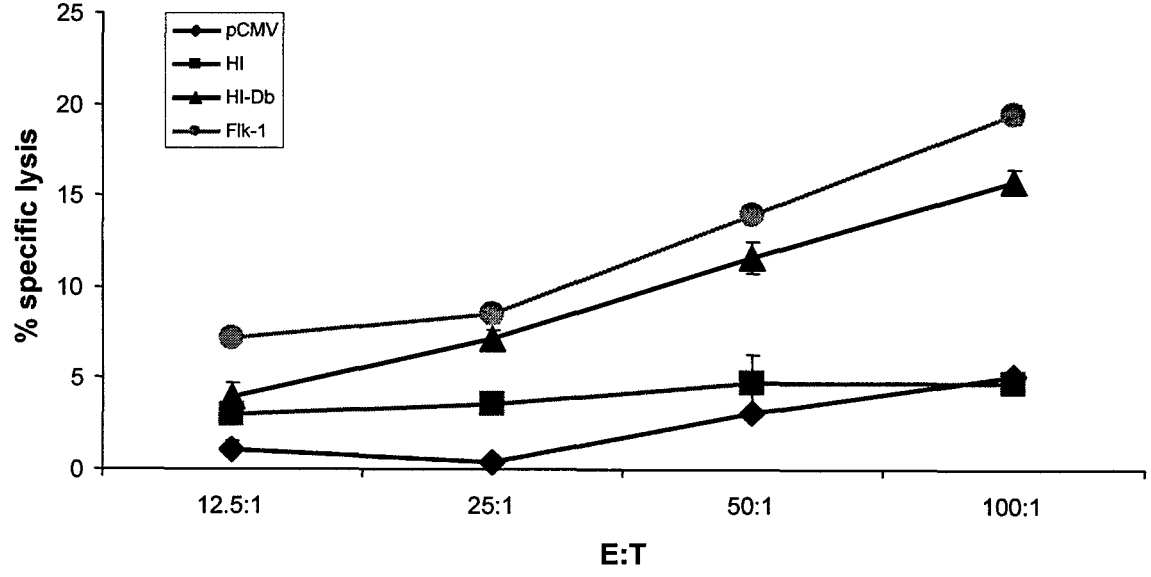


Fig. 9 T-cell mediated cytotoxicity against endothelial cell line MS1 by Flk-1-based vaccine

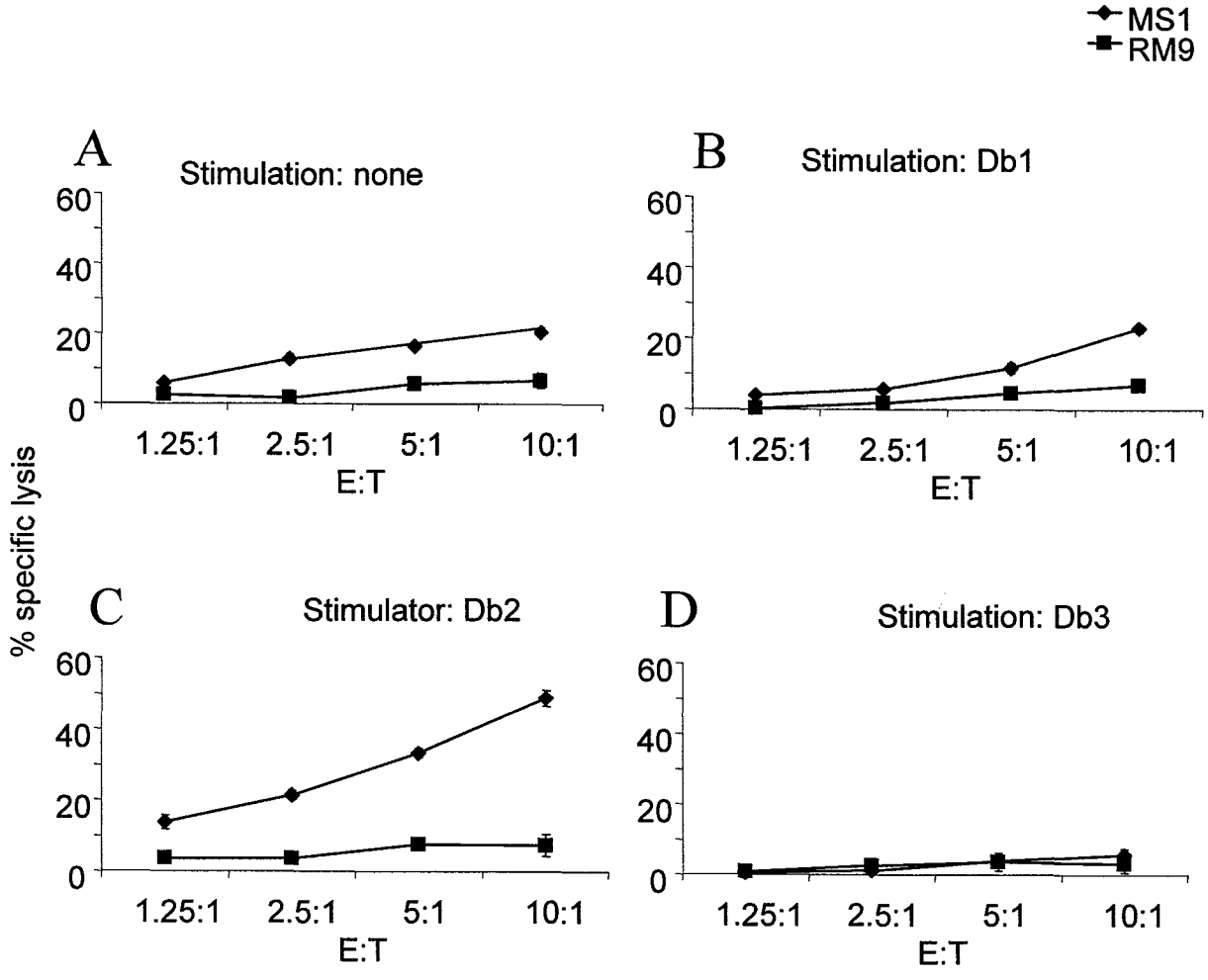


Fig 10 T-cell induced cytotoxicity against endothelial and tumor cells by each H-2D^b peptide-based vaccine

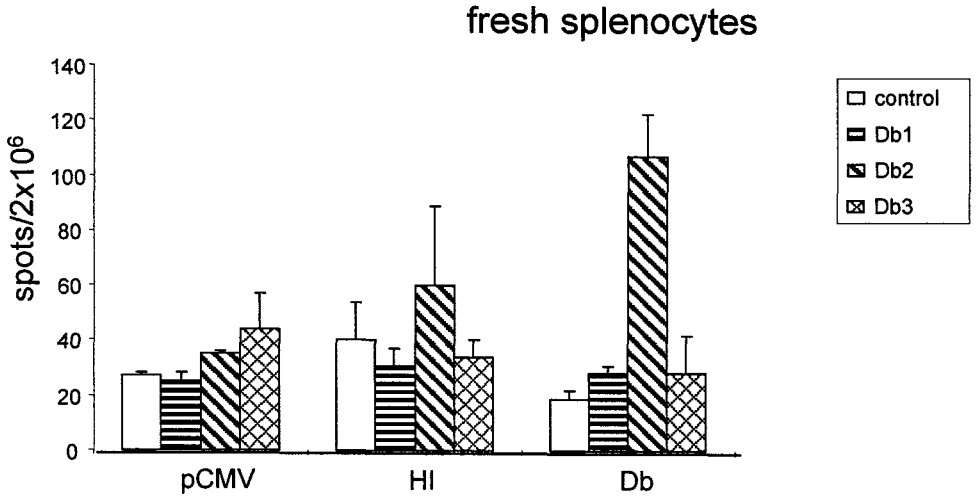


Fig. 11 Induction of IFN-γ at the Intracellular and Single T cell Level by the individual H-2D^b peptide

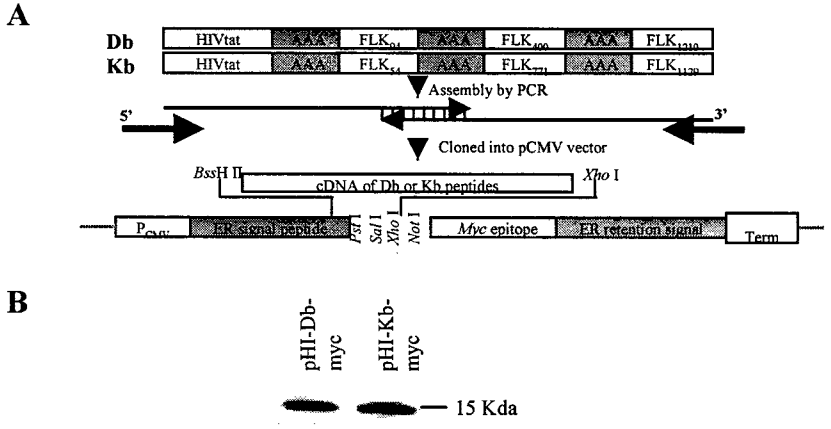


Fig 12 Construction of FLK-1 minigene DNA vaccine. (A) Schematic map. Minigenes encoding the HIVtat translocation peptide, a spacer, and murine FLK-1 H-2 Db-restricted and Kb-restricted epitopes were assembled by PCR with overlapping oligonucleotides as templates. Db-restricted epitopes include FLK_{94}: RVVGNDTGA; FLK_{400}: VILTNPISM; FLK1210: FHYDNTAGI. Kb-restricted epitopes include FLK_{54}: RGQRDLDWL; FLK_{771}: VIAMFFWLL; FLK_{1129}: TTPEMYQTM. The PCR fragments generated were cloned into a pCMV vector by using *Bss*H II and *Xho* I restriction sites. (B) Proteins encoded by minigenes were expressed in mammalian cells. This was indicated when 293T cells were transfected with either pHI-Db-myc or pHI-Kb-myc for 24 hours, harvested, lysed and analyzed by Western blotting with monoclonal antibody against myc.

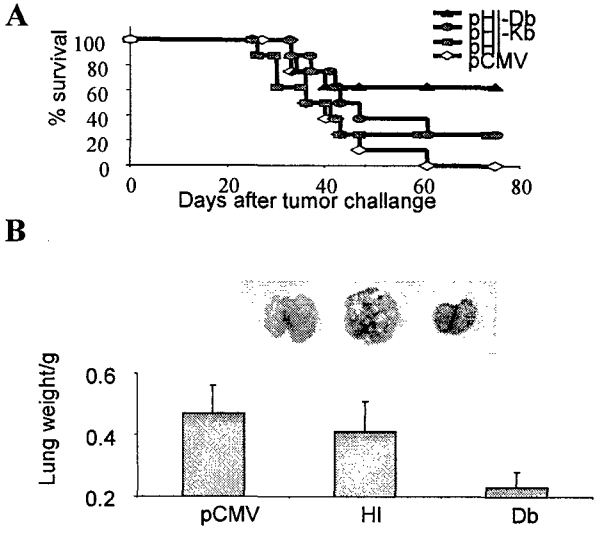


Fig. 13 DNA minigene vaccine pHI-Db protects mice from tumor cell challenge. Groups of C57BL/6 mice (n=8) were immunized 3 times at 1 wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Mice were challenged 2 wk after the last immunization i.v. with $1x10^5$ tumor cells. empty diamonds (◊) indicate pCMV control group; shaded squares (□) show pHI controul group; red triangles (Δ) depict pHI-Db group; while green circles (○) stand for pHI-Kb groups. (A) Survival study of mice challenged with D121 lung carcinoma cells. (B) Metastasis study of mice challenged with RM9 prostate carcinoma cells. Mice were sacrificed 28 days after tumor cell challenge, and lung weights assessed. Upper panel shows representative lungs, lower panel indicated average lung weight. Normal lung weight is about 0.2 g

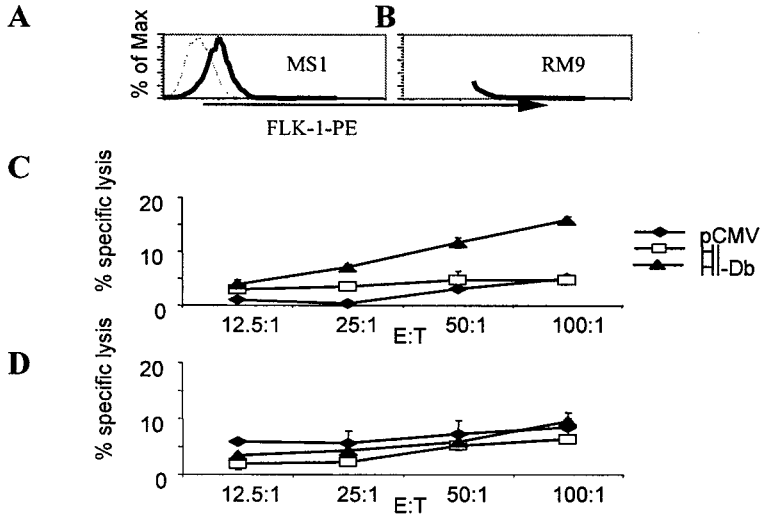


Fig. 14 The DNA minigene vaccine pHI-Db induces specific killing of FLK-1+ endothelial cells, but not FLK-1- tumor cells. Surface expression of FLK-1 by endothelial cell line MS1 (A) and murine prostate carcinoma cell line RM9 (B). Cells were washed and incubated with PE-conjugated isotype control Ab (thin dotted lines), or PE-conjugated anti-FLK-1 (thick solid lines). Groups of C57BL/6J mice (n=4) were immunized 3 times at 1 wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Mice were sacrificed 2 wk after the last immunization and isolated splenocytes were stimulated with irradiated MS1 cells for 5 d. Thereafter cytotoxicity assays were performed with MS1 (C) or RM9 (D) as target cells.

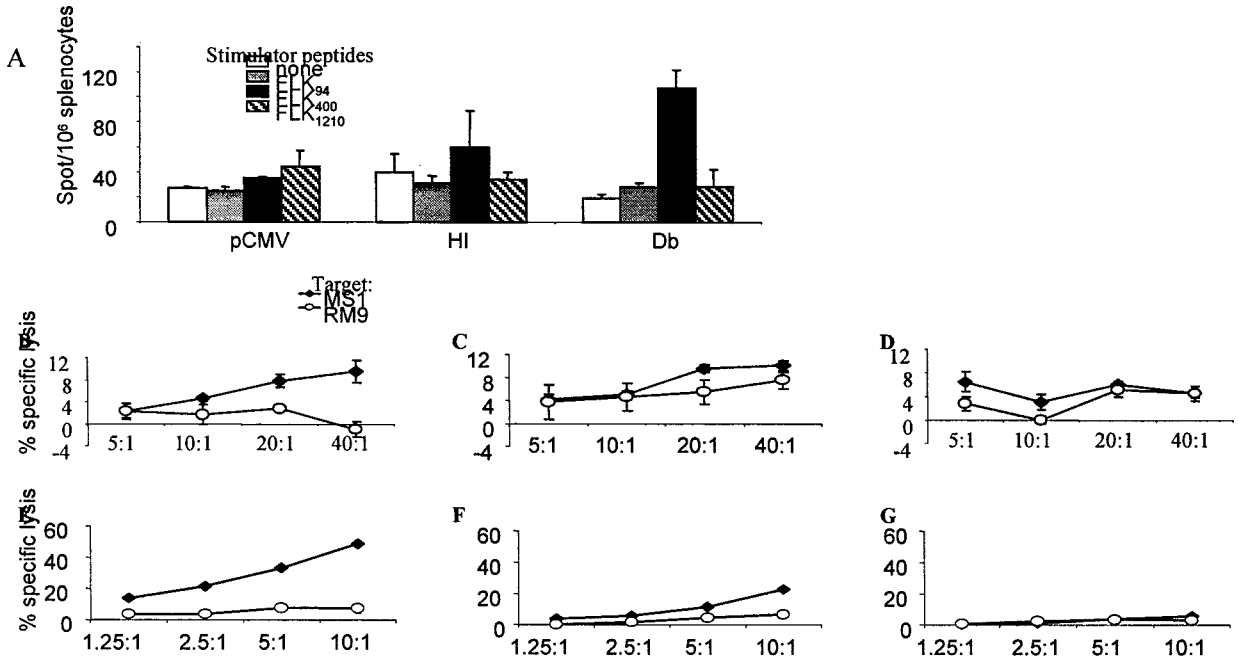


Fig. 15 An H-2 Db restricted FLK_{400}-specific response is induced by the pHI-Db DNA minigene vaccine. Groups of C57BL/6 mice (n=4) were immunized 3 times at 1 wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Two wk after the last immunization, mice were sacrificed. (A) ELISPOT assays were performed on splenocytes isolated by using no stimulator (empty bars) or synthetic peptides FLK_{94} (25 μg/ml, shaded bars), FLK_{400} (10 μg/ml, solid bars), or FLK_{1210} (25 μg/ml, striped bars) as stimulators. (B-D) Isolated splenocytes were stimulated with FLK_{94} (B), FLK_{400} (C) or FLK_{1210} (D) peptides for 5 d. Thereafter cytotoxicity assays were performed with MS1 (solid diamonds, ♦) or RM9 (empty circles, ○) as target cells. (E-G) Splenocytes isolated from pHI-Db vaccinated mice were stimulated with FLK_{94} (E), FLK_{400} (F) or FLK_{1210} (G) peptides for 7 d, and restimulated twice weekly with irradiated FLK_{94} (E)-, FLK_{400} (F)- or FLK_{1210} (G)-loaded splenocytes from normal C57BL/6 mice. Thereafter cytotoxicity assays were performed with MS1 (solid diamonds, ♦) or RM9 (empty circles, ○) as target cells.

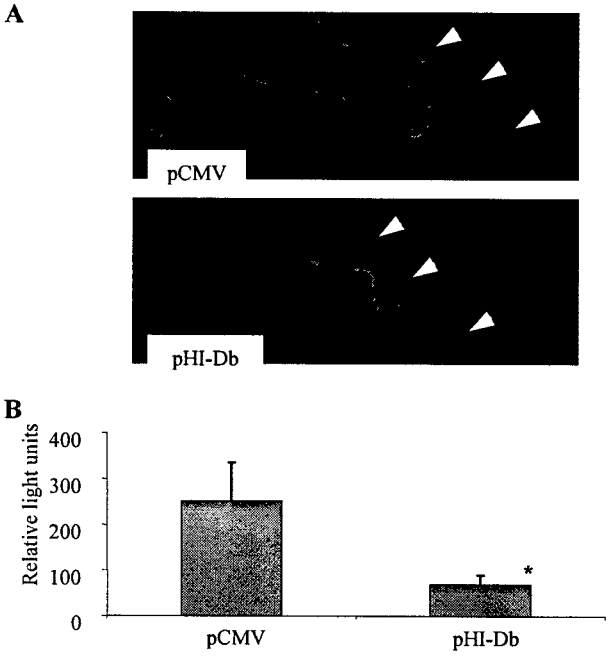


Fig. 16 Suppression of angiogenesis. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC-labeled isolectin B4. (A) Representative Matrigel plugs examined by con-focal microscopy (Magnification: x200). The arrows indicate the borders of the Matrigel plug. Matrigel was implanted into mice vaccinated with empty vector or pHI-Db. (B) The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs (n=4; mean + SD). Comparison of control group with treatment group indicated statistical significance ($P<0.02$).

Table 1: Predictions for H-2D^b/H-2K^b binding motifs

Nona-Motif	Rank	Position	Motif	Binding Score
H-2D^b	1	400	VILTNPISM	660.0
	4	1210	FHYDNTAGI	220.0
	15	94	RVVGNDTGA	36.0
H-2K^b	1	1129	TTPEMYQTM	86.4
	9	771	VIAMFFWLL	12.0
	18	54	RGQRDLDWL	4.75

Table 2. Effect of DNA vaccine encoding bFGF-R/secretory IL-15 on RM2 prostate cancer metastases

Experimental groups	Metastasis score
Empty vector	3, 3, 3, 3
hIL-15	1, 2, 3, 3
FGFR-long	0, 1, 1, 2
FGFR-long/hIL-15	0, 0, 0, 1

C57BL/6J mice were immunized with DNA vaccine encoding the long isoform of bFGF-r and 2 weeks later with the same vaccine additionally encoding secretory hIL-15. Two weeks thereafter these animals (n=4) were challenged by i.v. injection of 5 x10^4 RM-2 prostate cancer cells and then evaluated 4 weeks later for lung metastases. Since these were fused, the lung surface covered by metastases was evaluated as follows: 0=no metastases; 1=<5%, 2=5-25%; 3=>50%.

Table 3. Effect of DNA vaccine encoding FLK-1 and CD40TL against RM9 prostate cancer metastases in pulmonary experimental model

Exp. Groups[1]	Metastasis score	Lung weight
pEmpty vector	2,2,3,3,3,3,3	0.65± 0.12
pCD40LT	2,2,3,3,3,3,3,3	0.73± 0.15
pID/CD-FLK	0,1,2,2,2,2,3,3	0.48± 0.11
pID/ED-FLK	0,0,1,1,1,2,2,3	0.45± 0.14
pFLK1	0,0,1,1,1,1,2,3	0.33± 0.08
pFLK1+CD40TL	0,0,0,0,1,2,3,3	0.31± 0.11

1.CD40LT: pCD40 ligand trimer; pCD/ID: The plasmid encoding the cytoplasmic and intercellular domains of FLK-1gene; pED/ID: plasmid encoding the extracellular and intercellular domains of FLK-1. Fused metastases were evaluated by 5 lung surface covered as bellows: 1=< 5%; 2=5 to 25%; 3 >50%.